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FIBRONECTINS

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(57) Claim

1. A polypeptide having specific affinity for collagen or fibrin, wherein the collagen-binding polypeptide has the amino-acid sequence 379 to 445 shown in Figure 2 or a continuous collagen-binding part thereof and the fibrin-binding polypeptide has the amino-acid sequence 21 to 241 shown in Figure 2 or a continuous fibrin-binding part thereof and in either case the said polypeptide is not colinear with and adjacent the regions which are adjacent to and colinear with it in human fibronectin.

3. A method of purifying a substance which comprises contacting a conjugate of that substance and a collagen-binding polypeptide according to Claim 1 with immobilized collagen so that the said conjugate binds to the said collagen, and then eluting the said conjugate.

4. A method according to Claim 3 in which the said eluted conjugate is then split to remove the said collagen-binding polypeptide, and the said substance is then isolated.

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Complete Specification for the invention entitled:

"FIBRONECTINS"

The following statement is a full description of this invention, including the best method of performing it known to us :-

FIBRONECTINS

Fibronectins (FNs) constitute a class of high molecular weight glyco-proteins that have a key role in various contact processes of the vertebrates such as cell attachment and spreading, cell migration, control of cell morphology, differentiation and oncogenic transformation. All these biological activities imply interaction of FN with cells and with extracellular materials. Binding activities for collagen, heparin, fibrin, cell surfaces, bacteria and DNA have been located in different domains of the FN molecule (for review, see Yamada, 1983).

FN is one of the most versatile known proteins, both functionally and structurally. FN molecules are usually dimers of similar but not identical polypeptides of MW $\simeq$ 250,000. Cellular FN is found in a fibrilar component of the extracellular matrix of fibroblasts and other cell types. Plasma FN is a soluble molecule present in high concentrations in plasma (300μg/ml) and probably involved in opsonization, wound healing and haemostasis (Yamada, 1983; Hynes & Yamada, 1982). Partial primary structure data have revealed high conserved amino acid sequences both between the two FN forms and among FNs from different species: bovine plasma (Petersen et al, 1983), bovine cellular (Kornblihtt et al, 1983), human plasma (Pande & Shively 1982; Garcia-Pardo et al, 1983), human cellular (Kornblihtt et al, 1983, 1984a; Oldberg et al, 1983), rat plasma (Schwarzbauer et al, 1983). These data have tended to confirm that the basic FN polypeptide contains three different types of internal repeats (homology types I, II and III, approximately 40, 60 and 90 amino acids long respectively, as originally shown in bovine plasma FN (Skorstengaard et al, 1982; Petersen et al, 1983). Variations on this basic fibronectin structure account for the differences between cellular and plasma fibronectins and also between the polypeptide chains of both forms.

The diverse forms of fibronectin seem to be generated by transcription of a single gene into a common precursor which undergoes alternative splicing (Vibe-Pedersen et al, 1984). To-date, at least two regions have been described where this type of variation occurs. In certain human cell lines (fibroblasts, Hs578T) FN mRNAs can be distinguished by a 270 nucleotide segment (ED) that encodes exactly one of the homology type III. This ED segment seems to be absent in the liver hepatocyte mRNAs which are the source of plasma FN (Kornblihtt et al, 1983, 1984b). Schwarzbauer et al (1983) have reported three different FN mRNAs arising by alternative splicing in rat liver which differ in an area (IIICS) located to the 3' carboxy terminus side in the protein of the ED region. The difference sequence does not belong to any of the known internal homologies and it is inserted between the last two type III homology repeats, near the COOH terminus. In addition, Umezawa et al (1985) have reported further variations in the equivalent IIICS area of human liver FN mRNA, bringing the total to 5 alternative motifs for this area. The differences observed between FN polypeptides are thus the consequence of internal primary sequence variability (Kornblihtt et al, 1984a 1984b; Schwarzbauer et al, 1983), due to alternative splicing in at least two distinctive regions of the pre-mRNA (Tamkun et al, 1984; Vibe-Pedersen et al, 1984; Umezawa et al, 1985).

The complete amino acid sequence of mature human FN polypeptides has now been determined from the nucleotide sequence of multiple cDNA clones as described below. The polypeptide length varies from 2146 to 2325 amino acids, depending on which internal alternative splicing has taken place.

The present invention thus makes it possible to provide any desired part of the fibronectin molecule and in particular polypeptides having each of the separate binding activities of fibronectin separate from the others.

In the accompanying Figure 2, the binding sites of each part of the FN amino-acid sequence are given. While some of these were previously known, the sequence for the human collagen-binding site (lines 8-13 in the Figure) is new. Lines 9 and 10, involving the type II homology, are believed to be of particular significance, and to incorporate most or all of the collagen-binding ability.

The present invention thus provides novel polypeptides having substantially the amino acid sequence from 379 to 455 shown in Figure 2, or any continuous part thereof having collagen-binding activity. In practice such polypeptides may be linked to further amino-acid residues not affecting the desired end use of the collagen-binding polypeptide, including additional residues of the fibronectin molecule itself.

Similarly other sequences of the fibronectin molecule may be utilized for their ability to bind to other molecules. Thus, as shown in Figure 2, the polypeptide sequence from 21 to 241 is associated with binding to fibrin, heparin and <u>Staphylococcus aureus</u>. Other sequences are, as shown, associated with binding to DNA, cells and alternative heparin and fibrin binding sites.

The novel polypeptides may be made by culturing cells containing endogenous DNA coding for the polypeptide and separating the polypeptide from the metabolic products. Thus, the appropriate DNA sequences may be cloned into a competent strain of <u>E.coli</u> or other microorganism, e.g. a yeast such as <u>Saccharomyces cerevisiae</u>, the latter cultivated, and then the desired polypeptide isolated from the cultivation products. Figure 3 shows the complete DNA sequence for fibronectin and the associated amino-acid residues, and from this the cDNA sequence required for cloning the expression of any desired part of the fibronectin molecule may be easily

determined.

The DNA sequence coding for the collagen-binding polypeptide runs from coordinate 1147 to coordinate 1351, and the sequence coding for the fibrin-binding polypeptide runs from coordinate 73 to 738.

More particularly, however, in accordance with the present invention,

the clones herein described as pFH54, pFH134, pFH16 and pFH6 and similar clones may be used to produce corresponding polypeptides by expression in _E. coli_ or other appropriate microorganisms.pFH134 and pFH16 contain the DNA sequence for the collagen-binding part of the fibronectin molecule and may be used to generate a polypeptide having the collagen-binding activity of fibronectin without its other binding affinities. pFH6 can be used to transform competent _E. coli_ for the expression of a polypeptide binding fibrin and heparin.

The manner in which the clones pFH54, pFH134, pFH16 and pFH6 were obtained is described below, and repetition of the methods referred to will give similar clones with essentially the same or only slightly different utility. It will be appreciated in this connection that the isolation of useful cDNA sequences using the total cellular RNA from an appropriate source, i.e. cells naturally capable of expressing the desired protein or polypeptide, is a matter of routine experimentation for the person skilled in the art using the currently available techniques, particularly when, as is the case here, the actual amino acid sequence for the desired protein or polypeptide and the corresponding DNA sequence are both known. The experimental section below mentions appropriate techniques which have been found to be effective but it is to be expected that other known techniques would be equally applicable. Similarly choice of appropriate sources of RNA, vectors, and competent microorganisms for transformation from the many materials which are now available to the experimentalist is well within the ordinary skill in this art.

It will be appreciated that when a desired amino-acid sequence of fibronectin is expressed by a transformed microorganism it may be associated with a polypeptide characteristic of the microorganism itself. This may be immaterial to the intended use of the polypeptide but, in some cases, eg. if the polypeptide is to be used in therapy,

the presence of the additional amino-acid residues may be unacceptable. In that case the polypeptide must be subjected to an additional treatment, eg. with a protease, to separate the desired polypeptide free from undesired additional amino-acid residues.

As indicated above, the present invention is of especial interest in that it provides a means for transforming an appropriate microorganism to make it capable of expressing a polypeptide able to bind to collagen and/or fibrin. A polypeptide able to bind to collagen can be used, for example, to facilitate affinity purification of valuable polypeptides. Thus if the collagen-binding polypeptide is expressed in a form in which it is bound to another polypeptide of interest or if it is linked, after isolation, to such a polypeptide, the combined polypeptides may be purified by affinity chromatography on a column of bound gelatin (i.e. collagen), and then, after the purification, the desired polypeptide may be separated, eg. by an enzymatic hydrolysis, from the collagen-binding polypeptide.

A polypeptide able to bind to fibrin may be used in therapy to target a therapeutic agent on natural fibrin, eg. a blood clot. For example, a fibrinolytic enzyme bound to the polypeptide would have improved clot-dissolving properties, since it would have improved adhesion to its target.

In the accompanying drawings, <u>Fig. 1</u> shows a restriction enzyme map of seven cDNA clones covering 7692 nucleotides from the poly(A) tail of human FN mRNA. Human FN mRNA has been estimated to be 7900 nucleotides long (Kornblihtt <u>et al</u>, 1983). The clones cover the complete coding region for the mature protein (bottom diagram showing binding sites) and the 3' non-coding region. The dotted lines indicate segments that are absent from the corresponding cDNA clones but that must have been

synthesized in the first strand cDNA reaction and that were lost as a result of the failure of the Klenow enzyme to complete the second cDNA strand. Numbering on the map is in base pairs.

Fig. 2 shows the complete amino acid sequence of a human FN polypeptide. Residues 1 and 2325 are the NH_2 and COOH termini of the mature protein. The sequence was deduced from the nucleotide sequence (see Figure 3) of the cDNA clones depicted in Fig. 1. Alignment shows internal homologies. Gaps were introduced to maximize the homology. Identical residues within a type of homology are boxed. The cell recognition tetrapeptide RGDS (Pierschbacher & Ruoslahti, 1984) is underlined. Positions 17 (Ser), 21 (Cys) and 42 (Val) are reported as Cys, Ser and Ala respectively by Garcia-Pardo et al (1983). The FN polypeptide shown in this Figure has 2325 residues with a MW = 255,905. If the mass contributed by the carbohydrate side chains, estimated to be 9% of the protein mass (Yamada, 1983), is added, the molecular weight of this FN polypeptide would increase to approximately 279,000. This figure appears to be considerably higher than the weights of the FN monomers estimated by SDS-PAGE (230-250,000). The discrepancy could be explained by the poor resolution of the SDS gels in the range of high molecular weight proteins together with the lack of appropriate protein standards in that range.

Symbols are as follows: ■, free SH groups; ⅄, sites for carbohydrate side chains; Δ, cleavage site for chymotrypsin. ▲, cleavage site for plasmin. The multiple fibronectin polypeptides can be generated by all the possible permutations of the alternative splice regions in lines 26 and 30 (as explained below).

Fig. 3 shows the complete nucleotide sequence for the human FN polypeptide of Figure 2 deduced from the sequences of the cDNA clones of Figure 1.

Fig. 4 shows the variations of the FN primary structure.

A is the complete structure of the mature protein. Black boxes are type I homologies; dashed boxes, type II homologies;

empty boxes, type III homologies.

B and C show schematic representation of the different FN polypeptides that could arise by the translation of the multiple FN RNAs observed in the ED region (B) and in the IIICS region (C). The name of the cDNA clones representing the corresponding encoding mRNA species is shown to the right of each polypeptide. ^ indicates contiguity. λrlf2, 4 and 6 were isolated from a rat liver cDNA library (Schwarzbauer et al, 1983), pFHL1 and 8 from a human liver cDNA library, and pFH1 was isolated from the Hs578T cell line cDNA library (Kornblihtt et al, 1983). It will be noted that all the variations include the zones binding to collagen and fibrin.

Figure 5 shows, in more detail than Figure 1, part of the fibronectin protein indicating the position of the collagen-binding domain and internal homologies (I, II, and III). Below, the position and sizes (in base pairs) of the series of cDNAs used in the bacterial expression experiments described below is indicated. Only the restriction enzyme sites relevant to the cDNAs pXFN1-8 are shown. The flanking Hind III and Bam HI sites of pFH134 and pFH16 occur in the polylinker of the vector.

Figure 1 shows the restriction map of different cDNA clones covering the 3' non-coding region and the complete coding region for the mature protein of human FN mRNA. The isolation of clones pFH1, pFH111 and pFH154 has been previously described (Kornblihtt et al, 1983, 1984a) and nucleotide sequence and deduced amino acid sequence of the latter has been published before (Kornblihtt et al, 1984b). The clones pFH54, pFH134, pFH16 and pFH6 are new. Isolation of these four cDNA clones covering the 5' third of the map involved the synthesis of an oligonucleotide primer. The sequence (namely 5-GCTGAACCATTTGCTGAGC) of the primer was complementary to the mRNA sequence of a region close to the 5' end of clone pFH154. The oligonucleotide was used to prime reverse transcription of total RNA from Hs578T cells (Hackett et al, 1977) and a cDNA library was prepared as described below. The clones pFH54, pFH134, pFH6 and pFH16 were selected for further analysis. The complete nucleotide sequence of these clones was determined and comprised 7692 bp, of which

6972 bp correspond to the coding region and 720 bp to the 3' nontranslatable region and poly(A)tail. The sequence is included in the full DNA sequence for FN shown in Figure 3.

The amino acid sequence of human fibronectin deduced from the nucleotide sequence of the clones in Figure 1 is shown in Figure 2. The alignment in Figure 2 maximises internal homologies. The complete FN chain presents regions which have 3 different types of internal homologies (types I, II and III) (Petersen et al, 1983) and regions which have no homologous counterparts within the molecule. The latter are the NH_2-terminal and COOH-terminal segments and the internal connecting strands. From NH_2-to COOH- terminus, FN is formed by one 20-residue long NH_2-terminal segment (Fig. 2, line 1), five units of type I homology or fingers (lines 2 to 6), one connecting strand (line 7), one finger (line 8), two units of type II homology (lines 9 and 10), three fingers (line 11, 12 and 13), one unit of type III homology (line 14), one connecting strand (line 15), fourteen units of type III homology (lines 16 to 29, including the ED polypeptide), one connecting strand (IIICS, line 30), one unit of type III homology (line 31), one connecting strand (line 32), three fingers (lines 33, 34 and 35) and the COOH- terminal segment (line 36).

The primary structure of FN reflects a level of order and complexity not seen before in any other protein. The symmetry in the array of the 16 units of type III homology is particularly interesting. Two type III units (Fig. 2, lines 14 and 31) are separated by connecting strands (lines 15 and 30) from a central block, having the remaining fourteen in a juxtaposed way. The degree of homology within the type III units is very high. Three residues are conserved in all the units , viz Trp (Fig. 2, box with residue 599 at the top), Leu (box with residue 640 at the top) and Tyr (box with residue 646 at the top). The conserved residues are distributed in two peaks around the Trp and the Tyr, separated by a valley of non homology. It is believed that the degree of order and conservation in

the type III sequences must reflect particular constraints of the secondary structure of the central region of FN. This region is not stabilized by disulphide bridges since the only two Cys residues present in the type III sequences (positions 1201 and 2075 in Figure 2) have been shown to exist in a reduced form (Vibe-Pedersen et al, 1982; Smith et al, 1982).

Several binding activities have been assigned to different regions of the FN molecule (see Figs 1 and 2). However, only in the case of the ability to bind cells, has the actual binding site been identified so far. In fact, Pierschbacher and Ruoslahti (1984) demonstrated that the tetrapeptide Arg-Gly-Asp-Ser (RGDS) is responsible for the cell attachment activity of FN. This tetrapeptide is present only once in the sequence of Fig. 2 at positions 1493 to 1496, within one of the type III units. Fig. 2 also shows that the optimal alignment of the type III sequences in this area is obtained only if the tetrapeptide is considered to be an extra element, allowing four gaps in the corresponding regions of the rest of the type III units. It is probable that, as well as the cell binding site, other binding sites or biological activities within type III sequences reside in non-conserved stretches. The tetrapeptide has also been found in other proteins (Pierschbacher & Ruoslahti, 1984) including the α chain of fibrinogen which shows cell attachment activity.

An important feature of FN gene expression is the generation of slightly different polypeptides by differential processing of the common mRNA precursor (Vibe-Pedersen et al, 1984). Figure 4A shows diagrammatically the localization of the two regions of variability observed so far along the FN molecule (Schwarzbauer et al, 1983; Kornblihtt et al, 1984a 1984b). Figures 4B and 4C show the types of polypeptide that can arise from the translation of the different mRNAs generated in the ED (Fig. 2 line 26) and IIICS (Fig. 2, line 30) regions respectively. This diagram combines observations made on

both human and rat fibronectin. At least 10 different FN polypeptides can be generated from a single gene if it be assumed that all the permutations between the ED and IIICS segments are possible. This is consistent with the FN polypeptide heterogeneity observed in the bidimensional gel electrophoresis analysis of cellular and plasma FNs found in vivo. Homo- or hetero- dimeric FN molecules can then be formed from the FN polypeptide pool. The biological significance of this complex situation is not yet clear. However, it is to be noted that the ED and IIICS variable regions are intercalated between the cell-heparin and heparin-fibrin binding sites. The distance between these biologically active sites of the molecule may be critical for the FN function. For example, plasma FN is 1 to 2 orders of magnitude less active than cellular fibronectin in restoring morphology and alignment to a transformed fibroblast cell line (Yamada & Kennedy, 1979). Further the mRNAs carrying the ED segment are present in fibroblasts (one source of cellular FN) but not in liver cells (one source of plasma FN) (Kornblihtt et al, 1984b). It is possible that the function of the ED is to increase the distance between the cell binding tetrapeptide and the heparin binding site, resulting in an enhanced binding activity of the cellular FN molecule.

EXPERIMENTAL

RNA Preparation

Human cell line Hs578T (Hackett et al, 1977) was cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Total RNA was extracted from confluent cell monolayers by the guanidine-HCl method (Chirgwin et al, 1979). Between 2 and 4 mg of total RNA were extracted from 4×10^8 cells.

Other sources of RNA could be used if preferred, eg. fibroblasts or liver cells.

Isolation of fibronectin cDNA clones

All the cDNA clones depicted in Figure 1 were obtained using Hs578T cell RNA as template. Isolation of clone pFH1 by oligonucleotide probing was described by Kornblihtt et al (1983). Isolation of clones pFH111 and pFH154 by "mRNA walking" (oligonucleotide priming) was described by Kornblihtt et al (1984a). This latter procedure was used for the isolation of the new clones pFH54, pFH134, pFH16 and pFH6. An oligonucleotide primer complementary to the mRNA region close to the 5' end of pFH154 was synthesized by the method of Gait et al (1980). The oligonucleotide was used to prime reverse transcription of total RNA from Hs578T cells (Hackett et al, 1977). Blunt ended ds cDNA was prepared and cloned into the plasmid pAT153/PvuII/8 (Anson et al, 1984) in *E. coli* MC1061 as previously described (Kornblihtt et al, 1983). Colonies were screened using as probe a restriction fragment from the 5' end of pFH154 lacking the primer sequences, labelled by filling in at one end. In this way, clones pFH54 and pFH134 were obtained. In a second step, clones pFH16 and 6 were obtained by screening with an end labelled probe for the 5' end of clone pFH134.

Restriction fragments of the fibronectin cNDA were filled in with the Klenow fragment of DNA polymerase I and blunt end ligated into SmaI cut/phosphatased pEX 1, 2 or 3 vector. Transformations were carried out using the E. coli strain LKIII (Zabeau et al, 1982) harbouring the plasmid pcl857 which specifies kanamycin resistance and carries the cl857 allele (Remaut et al, 1983). Colonies were transferred to Whatman 541 filter paper (Gergen et al, 1985) and screened with either 3' end labelled (Maxam et al, 1977) or nick translated probes (Rigby et al, 1977).

Sequence determination

Inserts from clones were excised from the vector DNA by digestion with appropriate restriction enzymes, separated in agarose gel electrophoresis, and recovered by electroelution (Girwitz et al, 1980). Most of the sequencing was performed by the chemical degradation procedure of Maxam and Gilbert (1980). Some regions were sequenced by the chain terminator method (Sanger et al, 1977). For that purpose, the relevant fragments were isolated, digested either with AluI or HaeIII and ligated to a SmaI digested M13mp9 vector (Messing & Vieira, 1982), previously treated with calf intestinal phosphatase to prevent its circularization. The ligation mixtures were used to transform competent E. coli JM101 and recombinants were selected as clear plaques by insertional inactivation of the ß-galactosidase gene (Messing et al, 1981). Single stranded DNA was prepared by standard procedures (Winter & Fields, 1980) and the inserts were sequenced using a "universal" 17-nucleotide long primer (Duckworth et al, 1981).

Preparation of bacterial extracts

Bacteria carrying recombinant plasmids were grown at 30°C for 2½h and expression of the cro/ß-galactosidase fusion protein induced by shifting to 42°C for 2h. Bacteria were pelleted at 1200 g and washed with 50 mM TrisHCl, pH 7.4, 170 mM NaCl. Cells were resuspended in the same buffer containing lysozyme (2.5 mg/ml) and sonicated for 2 min on ice. The lysate was centrifuged at 45,000 g for 30 min at

4°C. The pellet was resuspended in 7 M urea in 10 mM Tris HCl, pH 7.4, 1mM EDTA and incubated at room temperature for 30 min. The solubilised extract was dialysed extensively against 50 mM Tris HCl pH 7.4 at 4°C, and then centrifuged at 45,000 g for 30 min at 4°C.

Gelatin-Sepharose chromatography

Gelatin-Sepharose was either obtained from Sigma Chemicals (St. Louis, MO, USA) or prepared by linking gelatin (pig skin type I, Sigma Chemicals) to CNBr-activated Sepharose CL.4B (Pharmacia, Uppsala, Sweden). Chromatography of bacterial extracts on gelatin-Sepharose was carried out as described by Ruoslahti *et al* (1982). The efficacy of the gelatin-Sepharose matrix was verified using purified human plasma fibronectin (Sigma Chemicals).

Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis was carried out in 0.1% (w/v) SDS in Tris/glycine buffer on 7.5% (w/v) acrylamide slab gels (19). Gels were stained with 0.1% Coomassie blue in methanol/water/acetic acid (4:5:1 by vol.). Immunoblotting was performed as described by Towbin et al. (1979). Polypeptides, electrophoretically transferred to nitrocellulose were probed with rabbit anti(human plasma fibronectin) serum (1:500 in phosphate buffered saline, 10% newborn calf serum and 0.05% Tween 20). Bound immunoglobulin was visualised using alkaline phosphatase conjugated goat anti-(rabbit IgG) (1:1000; Sigma Chemicals).

Protein assay

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Construction and characterisation of fibronectin expression plasmids

The human fibronectin cDNA clones, pFH134 and pFH16, encompass all or part of the collagen-binding domain of fibronectin identified by proteolytic cleavage of the protein (see Fig. 1 and Fig. 5). These cDNAs were therefore chosen as the starting point for investigating the expression of a functional collagen-binding site in *E. coli*. The pEX vectors

used for cloning enable exogenous gene sequences to be inserted into a polylinker in all three reading frames at the 3' end of a cro-LacZ hybrid gene under the control of the λ P_r promoter (Stanley et al, 1984). The 5' ends of the 1.74 kb and 1.04 kb inserts of pFH134 and pFH16 respectively were sequenced (Maxam et al, 1980) to establish the reading frames of the cDNAs and blunt end cloned into the SmaI site of pEX2. The recombinant plasmids were introduced into an E. coli strain previously transformed with a plasmid encoding the temperature-sensitive λP_r repressor, c1857. This allows for temperature-inducible expression of the cro/ß-galactosidase protein. To test for the production of fibronectin fusion protein by the expression constructs, hybridisation positive clones were grown at 30°C for 2½ h and then shifted to 42°C for a further 2 h. Total bacterial lysates were analysed by SDS poly-acrylamide gel electrophoresis. Five of ten pXFH134 constructs and one of seven pXFH16 constructs showed the production of high molecular weight polypeptides of sizes consistent with the lengths of the cDNA inserts (~ 185 kD and ~ 165 kD respectively. The correct orientation of the fibronectin sequences in pXFH134 and pXFH16 was confirmed by restriction enzyme analyses.

The fusion proteins produced by pXFH134 and pXFH16 accounted for approximately 20% of the total bacterial protein consistent with the previous report for this vector system (Stanley et al, 1984). Both fusion proteins showed some proteolytic degradation, particularly the pXFH134 polypeptide, which appeared to be partially cleaved to the size of the wild-type cro/ß-galactosidase (116 kD). Analysis of proteins synthesised over a time course of induction (0 to 120 min) indicated that proteolysis occurred concomitantly with synthesis of the fusion proteins.

The expression of fibronectin antigenic determinants in pXFH134 and pXFH16 was investigated by immunoblotting using a rabbit polyclonal anti-(human plasma fibronectin) serum.

The antiserum reacted with the 185 kD polypeptide synthesised by pXFH134 but not with the pXFH16 fusion protein or the cro/ß-galactosidase polypeptide, indicating that the epitope(s) recognised by the anti-serum lie outside the type II homology units and adjacent type I repeats (Fig. 5). This observation is consistent with the poor antigenicity of the collagen-binding domain of human fibronectin previously reported (Ruoslahti _et al_, 1979) and most probably reflects the very high level of amino acid conservation in this region (Fig. 2).

Gelatin-Sepharose affinity chromatography

Over-production of ß-galactosidase fusions in _E.coli_ results in the precipitation of the protein in the cells as insoluble inclusion bodies (Williams _et al_, 1982, Cheng 1983, Stanley 1983). Thus, when bacteria expressing the pXFH134 plasmid were lysed by sonication and centrifuged, the fibronectin fusion protein was found exclusively in the insoluble pellet. This fraction represented approximately 50% of the total protein of the bacterial lysate. Solubilisation of this material required treatment with 7 M urea and, following dialysis, 60% of the protein remained in solution. This fraction, which was highly-enriched in the fusion protein, was applied directly to a 5 ml gelatin-Sepharose column equilibrated in 50 mM Tris HCl, pH 7.4. The column was washed with 0.5 M NaCl in 50 mM Tris HCl, pH 7.4 until the E_{280} of the flowthrough was <0.01. The cro/ß-galactosidase-fibronectin hybrid protein was eluted from the column as a single symmetrical peak with 4 M urea in the same buffer. Under these conditions fibronectin is specifically released from gelatin-Sepharose (Ruoslahti _et al_, 1982). In the control experiment using pEX2 only, no binding of the wild-type cro/ß-galactosidase protein was observed.

A functional collagen-binding site has therefore been reconstituted in the pXFH134 fusion protein. It must be noted, however, that the fusion protein specifically eluted from the column represented < 5% of the fusion protein

applied to the column. Thus, not surprisingly, considerable activity is lost due to the insolubilisation of the fusion proteins in the bacterial cells, and subsequent vigorous treatment required to resolubilise them.

The fibronectin fusion protein produced by pXFH16 was also tested for gelatin-binding and showed similar activity to pXFH134. This indicated that the collagen-binding region occurred within the domain defined at the protein level and strongly implicated the two type II and adjacent type I homology units (see Fig. 5). To further localise the binding site, a series of overlapping expression constructs was made from pFH16 (Fig. 5) and systematically assayed for gelatin-binding activity. The results are summarised in Table 1, and show the consistent involvement of the type II homology units (pXFN 2, 3 and 6).

Table 1

pEX construct	Binding to Gelatin-Sepharose
pXFH134	+
pXFH16	+
pXFN1	-
2	+
3	+
4	-
5	-
6	+
7	-
8	-
pEX2 vector only	-

The binding activity of pXFH134 is almost entirely accounted for by a construct consisting of the two type II homology units (pXFN3). By comparing the gelatin-binding activity of pXFN3 and pXFN6 (both active) with pXFN5 and pXFN8 (both inactive), it may be deduced that the amino acid sequence critical for binding lies in the C-terminal half of the fibronectin fragment in pXFN3, and more particularly from the HinfI site (coordinate 1147 of Figure 3) to the RsaI site (coordinate 1351 of Figure 3). This 66 amino acid sequence represents almost the entire second type II homology unit of fibronectin plus a few amino acids of the adjacent type I homology unit (see Fig. 2).

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A polypeptide having specific affinity for collagen or fibrin, wherein the collagen-binding polypeptide has the amino-acid sequence 379 to 445 shown in Figure 2 or a continuous collagen-binding part thereof and the fibrin-binding polypeptide has the amino-acid sequence 21 to 241 shown in Figure 2 or a continuous fibrin-binding part thereof and in either case the said polypeptide is not colinear with and adjacent the regions which are adjacent to and colinear with it in human fibronectin.

2. A polypeptide according to Claim 1 bound to a polypeptide not present in human fibronectin.

3. A method of purifying a substance which comprises contacting a conjugate of that substance and a collagen-binding polypeptide according to Claim 1 with immobilized collagen so that the said conjugate binds to the said collagen, and then eluting the said conjugate.

4. A method according to Claim 3 in which the said eluted conjugate is then split to remove the said collagen-binding polypeptide, and the said substance is then isolated.

5. A polypeptide comprising a fibrin-binding amino-acid sequence according to Claim 1 bound to a therapeutic agent.

6. A cDNA compound coding for a polypeptide according to Claim 1.

7. A cDNA compound according to Claim 6 having the structure shown at 1147 to 1351 in Figure 3b.

8. A cDNA compound according to Claim 6 having the structure shown at 73 to 738 in Figure 3a.

9. A plasmid or other vector containing a cDNA compound as defined in any of Claims 6 to 8.

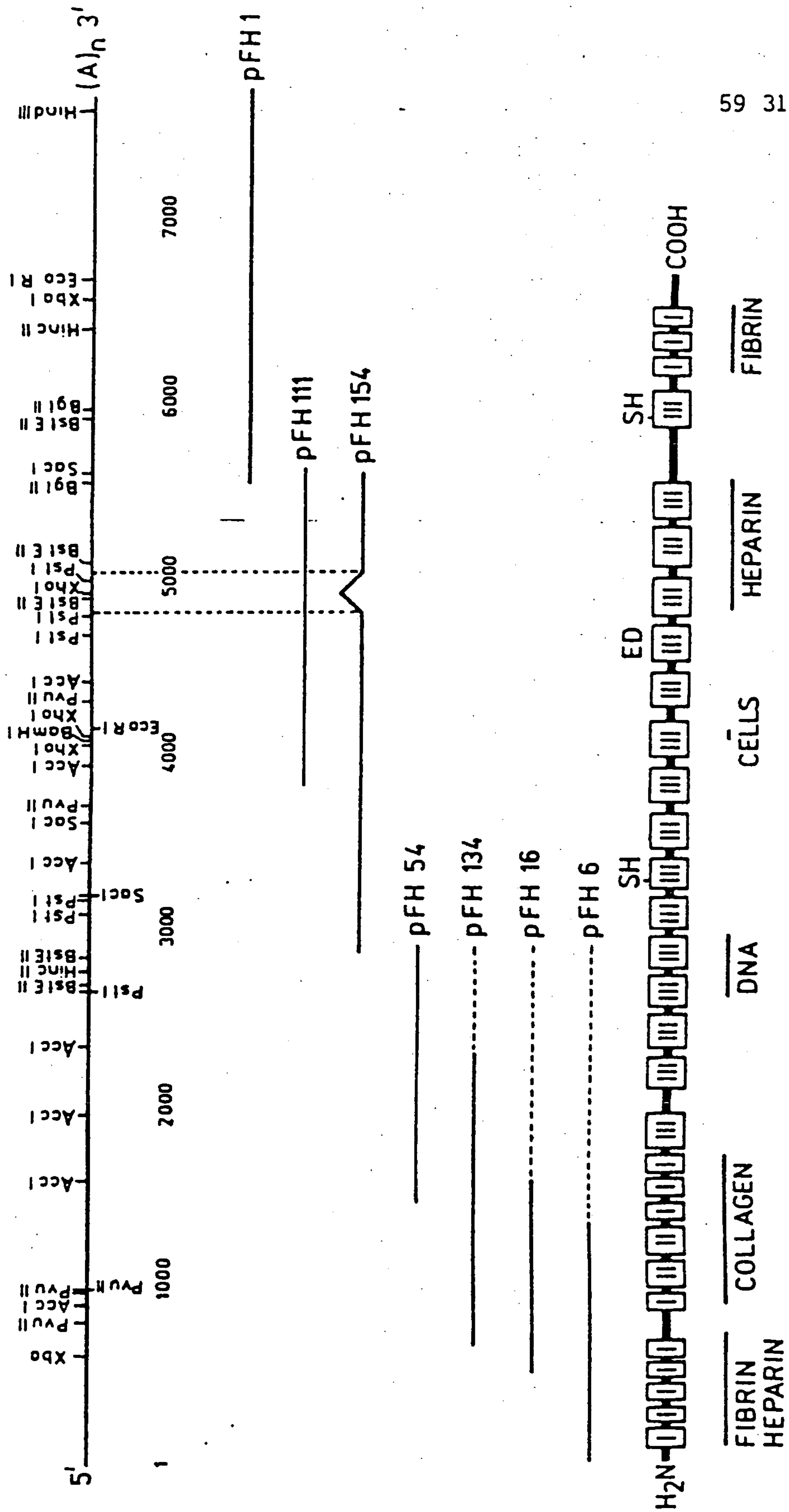
10. A microorganism modified by inclusion of a vector as claimed in Claim 9.

11. Escherichia coli modified by inclusion of a vector as claimed in Claim 9.

Dated this 1st day of August, 1990

DELTA BIOTECHNOLOGY LIMITED,
By its Patent Attorneys,
DAVIES & COLLISON

Fig. 1.


5'
(A)n 3'
1
1000
2000
3000
4000
5000
6000
7000
Xba I
Pvu II
Acc I
Acc I
Acc I
Acc I
BstE II
Hinc II
BstE II
Pst I
Pst I
Sac I
Acc I
Sac I
Pvu II
Acc I
Xho I
BamH I
Eco R I
Xho I
Pvu II
Acc I
Pst I
Pst I
BstE II
Xho I
Pst I
BstE II
Bgl II
Sac I
BstE II
Bgl II
Hinc II
Xba I
Eco R I
Hind III
pFH 1
pFH 111
pFH 154
pFH 54
pFH 134
pFH 16
pFH 6
H2N
COOH
SH
ED
SH
FIBRIN
HEPARIN
COLLAGEN
DNA
CELLS
HEPARIN
FIBRIN

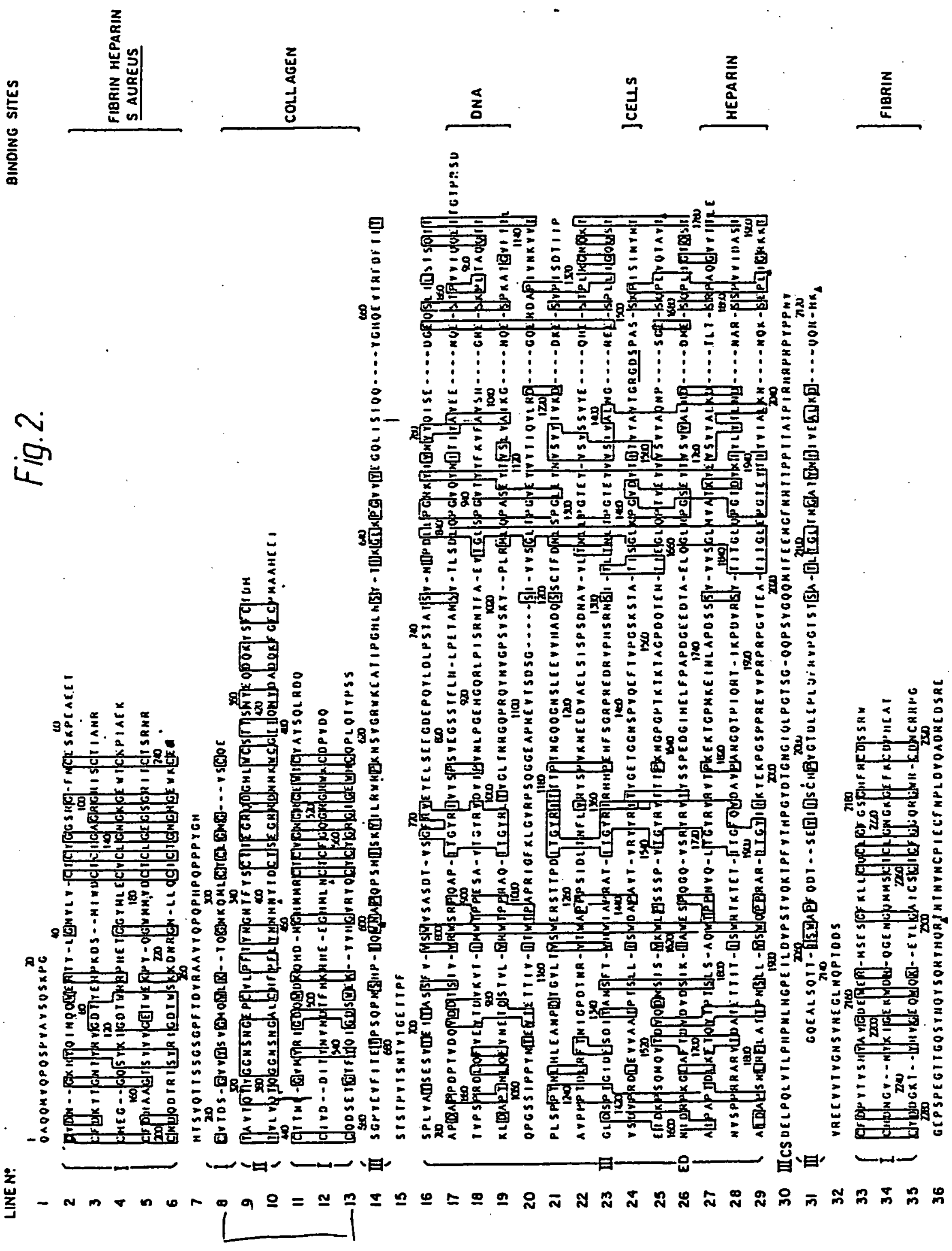

BINDING SITES
FIBRIN HEPARIN
S AUREUS
COLLAGEN
DNA
CELLS
HEPARIN
FIBRIN
LINE N°

Fig. 2.

Fig.3a.

FIRST SEQ. NO.=

1 (above Q), 2 (above C)
S K R Q A Q Q M V Q P Q S P V A V S Q S K P G C Y D N G K H Y Q I N Q Q W E R
GAAGAGCAAGAGGCAGGCTCAGCAAATGGTTCAGCCCCAGTCCCCGGTGGCTGTCAGTCAAAGCAAGCCCGGTTGTTATGACAATGGAAAACACTATCAGATAAATCAACAGTGGGAGCG
10 20 30 40 50 60 70 80 90 100 100 120

3
T Y L G N V L V C T C Y G G S R G F N C E S K P E A E E T C F D K Y T G N T Y R
GACCTACCTAGGTAATGTGTTGGTTTGTACTTGTTATGGAGGAAGCCGAGGTTTTAACTGCGAAAGTAAACCTGAAGCTGAAGAGACTTGCTTTGACAAGTACACTGGGAACACTTACCG
130 140 150 160 170 180 190 200 210 220 230 240

4
V G D T Y E R P K D S M I W D C T C I G A G R G R I S C T I A N R C H E G G Q S
AGTGGGTGACACTTATGAGCGTCCTAAAGACTCCATGATCTGGGACTGTACCTGCATCGGGGCTGGGCGAGGGAGAATAAGCTGTACCATCGCAAACCGCTGCCATGAAGGGGGTCAGTC
250 260 270 280 290 300 310 320 330 340 350 360

5
Y K I G D T W R R P H E T G G Y M L E C V C L G N G K G E W T C K P I A E K C F
CTACAAGATTGGTGACACCTGGAGGAGACCACATGAGACTGGTGGTTACATGTTAGAGTGTGTGTGTCTTGGTAATGGAAAAGGAGAATGGACCTGCAAGCCCATAGCTGAGAAGTGTTT
370 380 390 400 410 420 430 440 450 460 470 480

D H A A G T S Y V V G E T W E K P Y Q G W M M V D C T C L G E G S G R I T C T S
TGATCATGCTGCTGGGACTCCCTATGTGGTCGGAGAAACGTGGGAGAAGCCCTACCAAGGCTGGATGATGGTAGATTGTACTTGCCTGGGAGAAGGCAGCGGACGCATCACTTGCACTTC
490 500 510 520 530 540 550 560 570 580 590 600

6
R N R C N D Q D T R T S Y R I G D T W S K K D N R G N L L Q C I C T G N G R G E
TAGAAATAGATGCAACGATCAGGACACAAGGACATCCTATAGAATTTGAGACACCTGGAGCAAGAAGGATAATCGAGGAAACCTGCTCCAGTGCATCTGCACAGGCAACGGCCGAGGAGA
610 620 630 640 650 660 670 680 690 700 710 720

7
W K C E R H T S V Q T T S S G S G P F T D V R A A V Y Q P Q P H P Q P P P Y G H
GTGGAAGTGTGAGAGGCACACCTCTGTGCAGACCACATCGAGCGGATCTGGCCCCTTCACCGATGTTCGTGCAGCTGTTTACCAACCGCAGCCTCACCCCCAGCCTCCTCCCTATGGCCA
730 740 750 760 770 780 790 800 810 820 830 840

8, 9
C V T D S G V V Y S V G M Q W L K T Q G N K Q M L C T C L G N G V S C Q E T A V
CTGTGTCACAGACAGTGGTGTGGTCTACTCTGTGGGGATGCAGTGGTTGAAGACACAAGGAAATAAGCAAATGCTTTGCACGTGCCTGGGCAACGGAGTCAGCTGCCAAGAGACAGCTGT
850 860 870 880 890 900 910 920 930 940 950 960

Fig. 3b.

T Q T Y G G N L N G E P C V L P F T Y N G R T F Y S C T T E G R Q D G H L W C S
AACCCAGACTTACGGTGGCAACTTAAATGGAGAGCCATGTGTCTTACCATTCACCTACAATGGCAGGACGTTCTACTCCTGCACCACGGAAGGGCGACAGGACGGACATCTTTGGTGCAG
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080

10
T T S N Y E Q D Q K Y S F C T D H T V L V Q T Q G G N S N G A L C H F P F L Y N
CACAACTTCGAATTATGAGCAGGACCAGAAATACTCTTTCTGCACAGACCACACTGTTTTGGTTCAGACTCAAGGAGGAAATTCCAATGGTGCCTTGTGCCACTTCCCCTTCCTATACAA
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

N H N Y T D C T S E G R R D N M K W C G T T Q N Y D A D Q K F G F C P M A A H E
CAACCACAATTACACTGATTGCACTTCTGAGGGCAGAAGAGACAACATGAAGTGGTGTGGGACCACACAGAACTATGATGCCGACCAGAAGTTTGGGTTCTGCCCCATGGCTGCCCACGA
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

11
E I C T T N E G V M Y R I G D Q W D K Q H D M G H M M R C T C V G N G R G E W T
GGAAATCTGCACAACCAATGAAGGGGTCATGTACCGCATTGGAGATCAGTGGGATAAGCAGCATGACATGGGTCACATGATGAGGTGCACGTGTGTTGGGAATGGTCGTGGGGAATGGAC
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440

12
C I A Y S Q L R D Q C I V D D I T Y N V N D T F H K R H E E G H M L N C T C F G
ATGCATTGCCTACTCGCAACTTCGAGATCAGTGCATTGTTGATGACATCACTTACAATGTGAACGACACATTCCACAAGCGTCATGAAGAGGGGCACATGCTGAACTGTACATGCTTCGG
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560

13
Q G R G R W K C D P V D Q C Q D S E T G T F Y Q I G D S W E K Y V H G V R Y Q C
TCAGGGTCGGGGCAGGTGGAAGTGTGATCCCGTCGACCAATGCCAGGATTCAGAGACTGGGACGTTTTATCAAATTGGAGATTCATGGGAGAAGTATGTGCATGGTGTCAGATACCAGTG
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680

14
Y C Y G R G I G E W H C Q P L Q T Y P S S S G P V E V F I T E T P S Q P N S H P
CTACTGCTATGGCCGTGGCATTGGGGAGTGGCATTGCCAACCTTTACAGACCTATCCAAGCTCAAGTGGTCCTGTCGAAGTATTTATCACTGAGACTCCGAGTCAGCCCAACTCCCACCC
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800

I Q W N A P Q P S H I S K Y I L R W R P K N S V G R W K E A T I P G H L N S Y T
CATCCAGTGGAATGCACCACAGCCATCTCACATTTCCAAGTACATTCTCAGGTGGAGACCTAAAAATTCTGTAGGCCGTTGGAAGGAAGCTACCATACCAGGCCACTTAAACTCCTACAC
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 2000

Fig.3c.

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                                                                                 15
I K G L K P G V V Y E G Q L I S I Q Q Y G H Q E V T R F D F T T T S T S T P V T
CATCAAAGGCCTGAAGCCTGGTGTGGTATACGAGGGCCAGCTCATCAGCATCCAGCAGTACGGCCACCAAGAAGTGACTCGCTTTGACTTCACCACCACCAGCACCAGCACACCTGTGAC
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040

                    16
S N T V T G E T T P F S P L V A T S E S V T E I T A S S F V V S W V S A S D T V
CAGCAACACCGTGACAGGAGAGACGACTCCCTTTTCTCCTCTTGTGGCCACTTCTGAATCTGTGACCGAAATCACAGCCAGTAGCTTTGTGGTCTCCTGGGTCTCAGCTTCCGACACCGT
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160

S G F R V E Y E L S E E G D E P Q Y L D L P S T A T S V N I P D L L P G R K Y I
GTCGGGATTCCGGGTGGAATATGAGCTGAGTGAGGAGGGAGATGAGCCACAGTACCTGGATCTTCCAAGCACAGCCACTTCTGTGAACATCCCTGACCTGCTTCCTGGCCGAAAATACAT
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280

                                        17
V N V Y Q I S E D G E Q S L I L S T S Q T T A P D A P P D P T V D Q V D D T S I
TGTAAATGTCTATCAGATATCTGAGGATGGGGAGCAGAGTTTGATCCTGTCTACTTCACAAACAACAGCGCCTGATGCCCCTCCTGACCCGACTGTGGACCAAGTTGATGACACCTCAAT
2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400

V V R W S R P Q A P I T G Y R I V Y S P S V E G S S T E L N L P E T A N S V T L
TGTTGTTCGCTGGAGCAGACCCCAGGCTCCCATCACAGGGTACAGAATAGTCTATTCGCCATCAGTAGAAGGTAGCAGCACAGAACTCAACCTTCCTGAAACTGCAAACTCCGTCACCCT
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520

                                                                              18
S D L Q P G V Q Y N I T I Y A V E E N Q E S T P V V I Q Q E T T G T P R S D T V
CAGTGACTTGCAACCTGGTGTTCAGTATAACATCACTATCTATGCTGTGGAAGAAAATCAAGAAAGTACACCTGTTGTCATTCAACAAGAAACCACTGGCACCCCACGCTCAGATACAGT
2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640

P S P R D L Q F V E V T D V K V T I M W T P P E S A V T G Y R V D V I P V N L P
GCCCTCTCCCAGGGACCTGCAGTTTGTGGAAGTGACAGACGTGAAGGTCACCATCATGTGGACACCGCCTGAGAGTGCAGTGACCGGCTACCGTGTGGATGTGATCCCCGTCAACCTGCC
2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760

G E H G Q R L P I S R N T F A E V T G L S P G V T Y Y F K V F A V S H G R E S K
TGGCGAGCACGGGCAGAGGCTGCCCATCAGCAGGAACACCTTTGCAGAAGTCACCGGGCTGTCCCCTGGGGTCACCTATTACTTCAAAGTCTTTGCAGTGAGCCATGGGAGGGAGAGCAA
2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880
```

Fig. 3d.

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       19
P L T A Q Q T T K L D A P T N L Q F V N E T D S T V L V R W T P P R A Q I T G Y
GCCTCTGACTGCTCAACAGACAACCAAACTGGATGCTCCCACTAACCTCCAGTTTGTCAATGAAACTGATTCTACTGTCCTGGTGAGATGGACTCCACCTCGGGCCCAGATAACAGGATA
2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000

R L T V G L T R R G Q P R Q Y N V G P S V S K Y P L R N L Q P A S E Y T V S L V
CCGACTGACCGTGGGCCTTACCCGAAGAGGCCAGCCCAGGCAGTACAATGTGGGTCCCTCTGTCTTCAAGTACCCCCTGAGGAATCTGCAGCCTGCATCTGAGTACACCGTATCCCTCGT
3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120

                                20
A I K G N Q E S P K A T G V F T T L Q P G S S I P P Y N T E V T E T T I V I T W
GGCCATAAAGGGCAACCAAGAGAGCCCCAAAGCCACTGGAGTCTTTACCACACTGCAGCCTGGGAGCTCTATTCCACCTTACAACACCGAGGTGACTGAGACCACCATCGTGATCACATG
3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240

T P A P R I G F K L G V R P S Q G G E A P R E V T S D S G S I V V S G L T P G V
GACGCCTGCTCCAAGAATTGGTTTTAAGCTGGGTGTACGACCAAGCCAGGGAGGAGAGGCACCACGAGAAGTGACTTCAGACTCAGGAAGCATCGTTGTGTCCGGCTTGACTCCAGGAGT
3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360

                                            21
E Y V Y T I Q V L R D G Q E R D A P I V N K V V T P L S P P T N L H L E A N P D
AGAATACGTCTACACCATCCAAGTCCTGAGAGATGGACAGGAAAGAGATGCGCCAATTGTAAACAAAGTGGTGACACCATTGTCTCCACCAACAAACTTGCATCTGGAGGCAAACCCTGA
3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480

T G V L T V S W E R S T T P D I T G Y R I T T T P T N G Q Q G N S L E E V V H A
CACTGGAGTGCTCACAGTCTCCTGGGAGAGGAGCACCACCCCAGACATTACTGGTTATAGAATTACCACAACCCCTACAAACGGCCAGCAGGGAAATTCTTTGGAAGAAGTGGTCCATGC
3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600

                                                                            22
D Q S S C T F D N L S P G L E Y N V S V Y T V K D D K E S V P I S D T I I P A V
TGATCAGAGCTCCTGCACTTTTGATAACCTGAGTCCCGGCCTGGAGTACAATGTCAGTGTTTACACTGTCAAGGATGACAAGGAAAGTGTCCCTATCTCTGATACCATCATCCCAGCTGT
3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720

P P P T D L R F T N I G P D T M R V T W A P P P S I D L T N F L V R Y S P V K N
TCCTCCTCCCACTGACCTGCGATTCACCAACATTGGTCCAGACACCATGCGTGTCACCTGGGCTCCACCCCCATCCATTGATTTAACCAACTTCCTGGTGCGTTACTCACCTGTGAAAAA
3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 3840
```

Fig.3e.

```
E  E  D  V  A  E  L  S  I  S  P  S  D  N  A  V  V  L  T  N  L  L  P  G  T  E  Y  V  V  S  V  S  S  V  Y  E  Q  H  E  S
TGAGGAAGATGTTGCAGAGTTGTCAATTTCTCCTTCAGACAATGCAGTGGTCTTAACAAATCTCCTGCCTGGTACAGAATATGTAGTGAGTGTCTCCAGTGTCTACGAACAACATGAGAG
     3850      3860      3870      3880      3890      3900      3910      3920      3930      3940      3950      3960

                     23
T  P  L  R  G  R  Q  K  T  G  L  D  S  P  T  G  I  D  F  S  D  I  T  A  N  S  F  T  V  H  W  I  A  P  R  A  T  I  T  G
CACACCTCTTAGAGGAAGACAGAAAACAGGTCTTGATTCCCCAACTGGCATTGACTTTTCTGATATTACTGCCAACTCTTTTACTGTGCACTGGATTGCTCCTCGAGCCACCATCACTGG
     3970      3980      3990      4000      4010      4020      4030      4040      4050      4060      4070      4080

Y  R  I  R  H  H  P  E  H  F  S  G  R  P  R  E  D  R  V  P  H  S  R  N  S  I  T  L  T  N  L  T  P  G  T  E  Y  V  V  S
CTACAGGATCCGCCATCATCCCGAGCACTTCAGTGGGAGACCTCGAGAAGATCGGGTGCCCCACTCTCGGAATTCCATCACCCTCACCAACCTCACTCCAGGCACAGAGTATGTGGTCAG
     4090      4100      4110      4120      4130      4140      4150      4160      4170      4180      4190      4200

                                                24
I  V  A  L  N  G  R  E  E  S  P  L  L  I  G  Q  Q  S  T  V  S  D  V  P  R  D  L  E  V  V  A  A  T  P  T  S  L  L  I  S
CATCGTTGCTCTTAATGGCAGAGAGGAAAGTCCCTTATTGATTGGCCAACAATCAACAGTTTCTGATGTTCCGAGGGACCTGGAAGTTGTTGCTGCGACCCCCACCAGCCTACTGATCAG
     4210      4220      4230      4240      4250      4260      4270      4280      4290      4300      4310      4320

W  D  A  P  A  V  T  V  R  Y  Y  R  I  T  Y  G  E  T  G  G  N  S  P  V  Q  E  F  T  V  P  G  S  K  S  T  A  T  I  S  G
CTGGGATGCTCCTGCTGTCACAGTGAGATATTACAGGATCACTTACGGAGAAACAGGAGGAAATAGCCCTGTCCAGGAGTTCACTGTGCCTGGGAGCAAGTCTACAGCTACCATCAGCGG
     4330      4340      4350      4360      4370      4380      4390      4400      4410      4420      4430      4440

                                                                                                      25
L  K  P  G  V  D  Y  T  I  T  V  Y  A  V  T  G  R  G  D  S  P  A  S  S  K  P  I  S  I  N  Y  R  T  E  I  D  K  P  S  Q
CCTTAAACCTGGAGTTGATTATACCATCACTGTGTATGCTGTCACTGGCCGTGGAGACAGCCCCGCAAGCAGCAAGCCAATTTCCATTAATTACCGAACAGAAATTGACAAACCATCCCA
     4450      4460      4470      4480      4490      4500      4510      4520      4530      4540      4550      4560

M  Q  V  T  D  V  Q  D  N  S  I  S  V  K  W  L  P  S  S  S  P  V  T  G  Y  R  V  T  T  T  P  K  N  G  P  G  P  T  K  T
GATGCAAGTGACCGATGTTCAGGACAACAGCATTAGTGTCAAGTGGCTGCCTTCAAGTTCCCCTGTTACTGGTTACAGAGTAACCACCACTCCCAAAAATGGACCAGGACCAACAAAAAC
     4570      4580      4590      4600      4610      4620      4630      4640      4650      4660      4670      4680

K  T  A  G  P  D  Q  T  E  M  T  I  E  G  L  Q  P  T  V  E  Y  V  V  S  V  Y  A  Q  N  P  S  G  E  S  Q  P  L  V  Q  T
TAAAACTGCAGGTCCAGATCAAACAGAAATGACTATTGAAGGCTTGCAGCCCACAGTGGAGTATGTGGTTAGTGTCTATGCTCAGAATCCAAGCGGAGAGAGTCAGCCTCTGGTTCAGAC
     4690      4700      4710      4720      4730      4740      4750      4760      4770      4780      4790      4800
```

Fig. 3f.

26
A V T N I D R P K G L A F T D V D V D S I K I A W E S P Q G Q V S R Y R V T Y S
TGCAGTAACCAACATTGATCGCCCTAAAGGACTGGCATTCACTGATGTGGATGTCGATTCCATCAAAATTGCTTGGGAAAGCCCACAGGGGCAAGTTTCCAGGTACAGGGTGACCTACTC
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920

S P E D G I H E L F P A P D G E E D T A E L Q G L R P G S E Y T V S V V A L H D
GAGCCCTGAGGATGGAATCCATGAGCTATTCCCTGCACCTGATGGTGAAGAAGACACTGCAGAGCTGCAAGGCCTCAGACCGGGTTCTGAGTACACAGTCAGTGTGGTTGCCTTGCACGA
4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040

27
D M E S Q P L I G T Q S T A I P A P T D L K F T Q V T P T S L S A Q W T P P N V
TGATATGGAGAGCCAGCCCCTGATTGGAACCCAGTCCACAGCTATTCCTGCACCAACTGACCTGAAGTTCACTCAGGTCACACCCACAAGCCTGAGCGCCCAGTGGACACCACCCAATGT
5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160

Q L T G Y R V R V T P K E K T G P M K E I N L A P D S S S V V V S G L M V A T K
TCAGCTCACTGGATATCGAGTGCGGGTGACCCCCAAGGAGAAGACCGGACCAATGAAAGAAATCAACCTTGCTCCTGACAGCTCATCCGTGGTTGTATCAGGACTTATGGTGGCCACCAA
5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280

28
Y E V S V Y A L K D T L T S R P A Q G V V T T L E N V S P P R R A R V T D A T E
ATATGAAGTGAGTGTCTATGCTCTTAAGGACACTTTGACAAGCAGACCAGCTCAGGGTGTTGTCACCACTCTGGAGAATGTCAGCCCACCAAGAAGGGCTCGTGTGACAGATGCTACTGA
5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400

T T I T I S W R T K T E T I T G F Q V D A V P A N G Q T P I Q R T I K P D V R S
GACCACCATCACCATTAGCTGGAGAACCAAGACTGAGACGATCACTGGCTTCCAAGTTGATGCCGTTCCAGCCAATGGCCAGACTCCAATCCAGAGAACCATCAAGCCAGATGTCAGAAG
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520

29
Y T I T G L Q P G T D Y K I Y L Y T L N D N A R S S P V V I D A S T A I D A P S
CTACACCATCACAGGTTTACAACCAGGCACTGACTACAAGATCTACCTGTACACCTTGAATGACAATGCTCGGAGCTCCCCTGTGGTCATCGACGCCTCCACTGCCATTGATGCACCATC
5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640

N L R F L A T T P N S L L V S W Q P P R A R I T G Y I I K Y E K P G S P P R E V
CAACCTGCGTTTCCTGGCCACCACACCCAATTCCTTGCTGGTATCATGGCAGCCGCCACGTGCCAGGATTACCGGCTACATCATCAAGTATGAGAAGCCTGGGTCTCCTCCCAGAGAAGT
5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760

Fig.3g.

V P R P R P G V T E A T I T G L E P G T E Y T I Y V I A L K N N Q K S E P L I G
GGTCCCTCGGCCCCGCCCTGGTGTCACAGAGGCTACTATTACTGGCCTGGAACCGGGAACCGAATATACAATTTATGTCATTGCCCTGAAGAATAATCAGAAGAGCGAGCCCCTGATTGG
5770 5780 5790 5800 5810 5820 5830 5840 5850 5860 5870 5880

30
R K K T D E L P Q L V T L P H P N L H G P E I L D V P S T V Q K T P F V T H P G
AAGGAAAAAGACAGACGAGCTTCCCCAACTGGTAACCCTTCCACACCCCAATCTTCATGGACCAGAGATCTTGGATGTTCCTTCCACAGTTCAAAAGACCCCTTTCGTCACCCACCCTGG
5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000

Y D T G N G I Q L P G T S G Q Q P S V G Q Q M I F E E H G F R R T T P P T T A T
GTATGACACTGGAAATGGTATTCAGCTTCCTGGCACTTCTGGTCAGCAACCCAGTGTTGGGCAACAAATGATCTTTGAGGAACATGGTTTTAGGCGGACCACACCGCCCACAACGGCCAC
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120

31
P I R H R P R P Y P P N V G Q E A L S Q T T I S W A P F Q D T S E Y I I S C H P
CCCCATAAGGCATAGGCCAAGACCATACCCGCCGAATGTAGGACAAGAAGCTCTCTCTCAGACAACCATCTCATGGGCCCCATTCCAGGACACTTCTGAGTACATCATTTCATGTCATCC
6130 6140 6150 6160 6170 6180 6190 6200 6210 6220 6230 6240

V G T D E E P L Q F R V P G T S T S A T L T G L T R G A T Y N I I V E A L K D Q
TGTTGGCACTGATGAAGAACCCTTACAGTTCAGGGTTCCTGGAACTTCTACCAGTGCCACTCTGACAGGCCTCACCAGAGGTGCCACCTACAACATCATAGTGGAGGCACTGAAAGACCA
6250 6260 6270 6280 6290 6300 6310 6320 6330 6340 6350 6360

32 33
Q R H K V R E E V V T V G N S V N E G L N Q P T D D S C F D P Y T V S H Y A V G
GCAGAGGCATAAGGTTCGGGAAGAGGTTGTTACCGTGGGCAACTCTGTCAACGAAGGCTTGAACCAACCTACGGATGACTCGTGCTTTGACCCCTACACAGTTTCCCATTATGCCGTTGG
6370 6380 6390 6400 6410 6420 6430 6440 6450 6460 6470 6480

34
D E W E R M S E S G F K L L C Q C L G F G S G H F R C D S S R W C H D N G V N Y
AGATGAGTGGGAACGAATGTCTGAATCAGGCTTTAAACTGTTGTGCCAGTGCTTAGGCTTTGGAAGTGGTCATTTCAGATGTGATTCATCTAGATGGTGCCATGACAATGGTGTGAACTA
6490 6500 6510 6520 6530 6540 6550 6560 6570 6580 6590 6600

35
K I G E K W D R Q G E N G Q M M S C T C L G N G K G E F K C D P H E A T C Y D D
CAAGATTGGAGAGAAGTGGGACCGTCAGGGAGAAAATGGCCAGATGATGAGCTGCACATGTCTTGGGAACGGAAAAGGAGAATTCAAGTGTGACCCTCATGAGGCAACGTGTTACGATGA
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720

Fig.3h.

G K T Y H V G E Q W Q K E Y L G A I C S C T C F G G Q R G W R C D N C R R P G G
TGGGAAGACATACCACGTAGGAGAACAGTGGCAGAAGGAATATCTCGGTGCCATTTGCTCCTGCACATGCTTTGGAGGCCAGCGGGGCTGGCGCTGTGACAACTGCCGCAGACCTGGGGG
6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840

E P S P E G T T G Q S Y N Q Y S Q R Y H Q R T N T N V N C P I E C F M P L D V Q
TGAACCCAGTCCCGAAGGCACTACTGGCCAGTCCTACAACCAGTATTCTCAGAGATACCATCAGAGAACAAACACTAATGTTAATTGCCCAATTGAGTGCTTCATGCCTTTAGATGTACA
6850 6860 6870 6880 6890 6900 6910 6920 6930 6940 6950 6960

A D R E D S R E
GGCTGACAGAGAAGATTCCCGAGAGTAAATCATCTTTCCAATCCAGAGGAACAAGCATGTCTCTCTGCCAAGATCCATCTAAACTGGAGTGATGTTAGCAGACCCAGCTTAGAGTTCTTC
6970 6980 6990 7000 7010 7020 7030 7040 7050 7060 7070 7080

TTTCTTTCTTAAGCCCTTTGCTCTGGAGGAAGTTCTCCAGCTTCAGCTCAACTCACAGCTTCTCCAAGCATCACCCTGGGAGTTTCCTGAGGGTTTTCTCATAAATGAGGGCTGCACATT
7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200

GCCTGTTCTGCTTCGAAGTATTCAATACCGCTCAGTATTTTAAATGAAGTGATTCTAAGATTTGGTTTGGGATCAATAGGAAAGCATATGCAGCCAACCAAGATGCAAATGTTTTGAAAT
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320

GATATGACCAAAATTTTAAGTAGGAAAGTCACCCAAACACTTCTGCTTTCACTTAAGTGTCTGGCCCCGCAATACTGTAGGAACAAGCATGATCTTGTTACTGTGATATTTTAAATATCCA
7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440

CAGTACTCACTTTTTCCAAATGATCCTAGTAATTGCCTAGAAATATCTTTCTCTTACCTGTTATTTATCAATTTTTCCCAGTATTTTTATACGGAAAAAATTGTATTGAAAACACTTAGT
7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560

ATGCAGTTGATAAGAGGAATTTGGTATAATTATGGTGGGTGATTATTTTTTATACTGTATGTGCCAAAGCTTTACTACTGTGGAAAGACAACTGTTTTAATAAAAGATTTACATTCCACA
7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680

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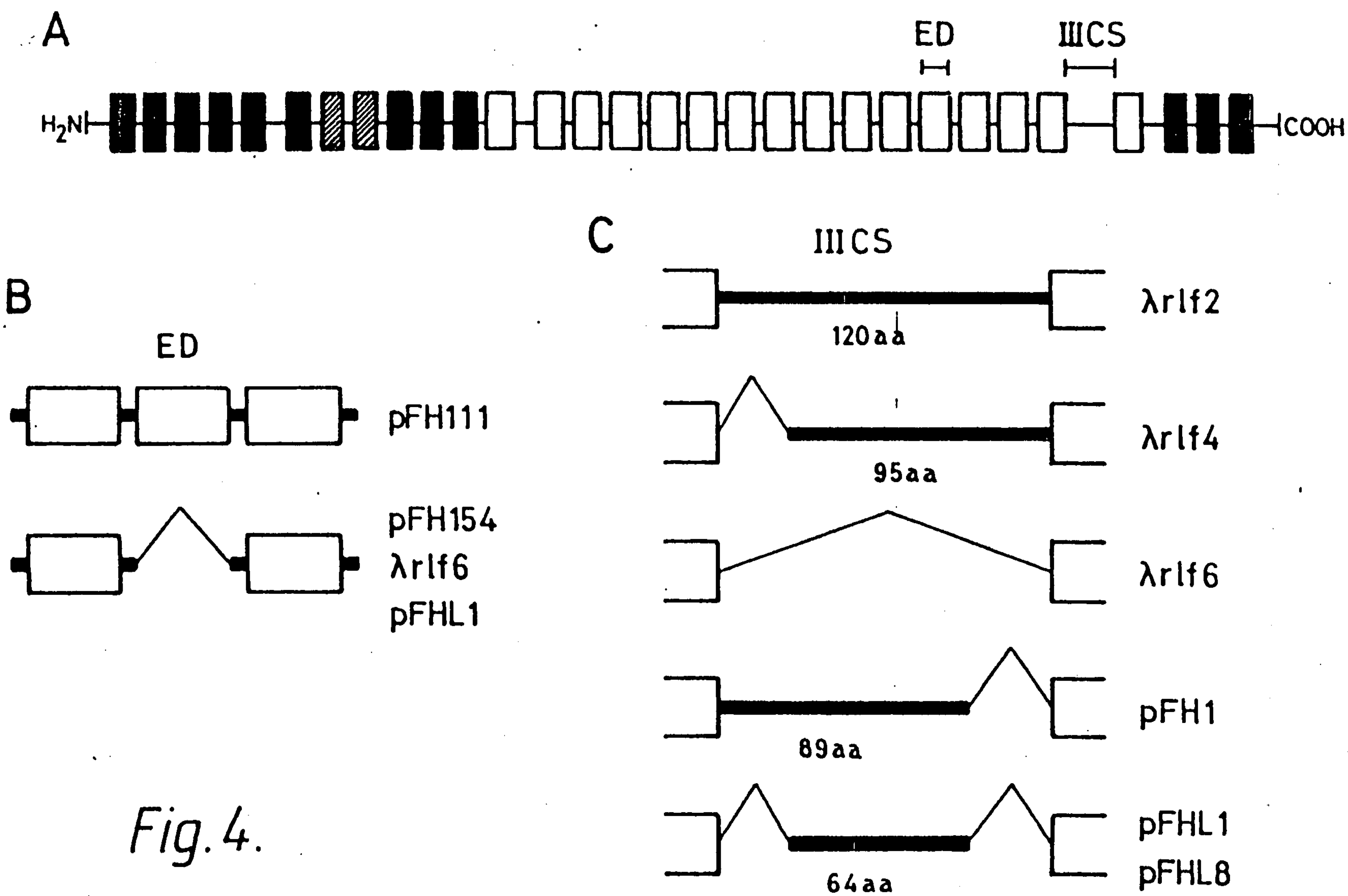
7690 7700 5 15 25 35 45 55 65 75 85 95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B
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λrlf6
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C
IIICS
120aa
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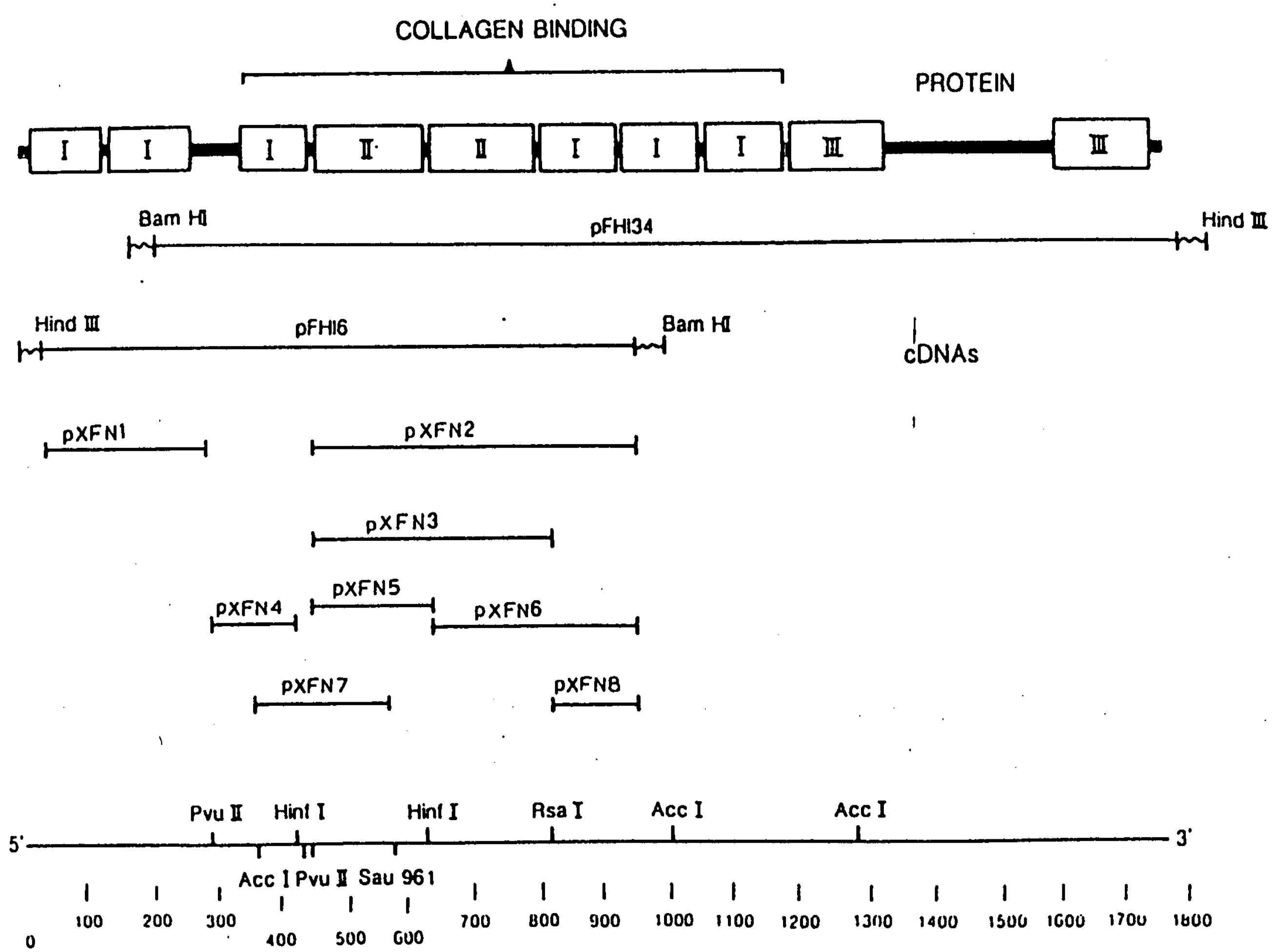
95aa
λrlf4
λrlf6
89aa
pFH1
64aa
pFHL1
pFHL8

Fig. 4.

Fig.5.



COLLAGEN BINDING
PROTEIN
I
I
I
II
II
I
I
I
III
III
Bam HI
pFHI34
Hind III
Hind III
pFHI6
Bam HI
cDNAs
pXFN1
pXFN2
pXFN3
pXFN4
pXFN5
pXFN6
pXFN7
pXFN8
Pvu II
Hinf I
Hinf I
Rsa I
Acc I
Acc I
5'
3'
Acc I
Pvu II
Sau 961
0
100
200
300
400
500
600
700
800
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1400
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1700
1800